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# Pathogenesis of *Mycobacterium avium* subsp. *paratuberculosis* in neonatal calves after oral or intraperitoneal experimental infection

J.R. Stabel [a,*], M.V. Palmer [a], B. Harris [b], B. Plattner [c], J. Hostetter [c], S. Robbe-Austerman [a]

[a] USDA-ARS, National Animal Disease Center, Ames, IA 50010, United States
[b] USDA-APHIS, National Veterinary Services Laboratory, Ames, IA 50010, United States
[c] Iowa State University, College of Veterinary Medicine, Ames, IA 50010, United States

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ABSTRACT

Understanding the host response to *Mycobacterium avium* subsp. *paratuberculosis* is critical to the development of effective vaccines and therapeutics for the control of this disease in the field. The current study compared the effectiveness of oral and intraperitoneal (IP) methods of experimental inoculation and two strains of *M. avium* subsp. *paratuberculosis* (strain K-10 and clinical isolate 509) on the level of infection and lesion development. Calves were inoculated with $4 \times 10^{11}$ to $8 \times 10^{12}$ cfu live bacteria, depending upon treatment group. Fecal shedding of *M. avium* subsp. *paratuberculosis* was minimal and infrequent over the course of the study for calves that received strain K-10 (oral and IP), however, calves orally inoculated with the clinical isolate shed high numbers of bacteria in their feces up to 4 months post-inoculation. Colonization was present in a number of intestinal tissues and lymph nodes with the lowest number of affected tissues in the IP calves and the highest for calves receiving the clinical isolate via oral inoculation. Microscopic lesions were predominantly found in the ileal and jejunal sections of small intestine and their associated lymph nodes, as well as the ileocecal valve and node. These data suggest that a variety of experimental infection regimes can be effective but oral inoculation with a clinical isolate may result in greater colonization of tissues and fecal shedding of *M. avium* subsp. *paratuberculosis*.

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## 1. Introduction

A successful animal model that mimics the natural disease state is a valuable resource as a testing platform for vaccine candidates and therapeutic agents. Current vaccine preparations for paratuberculosis do not prevent infection but do reduce fecal shedding and clinical disease thereby slowing the spread of disease (Larsen et al., 1978; Wentink et al., 1994; Uzonna et al., 2003). In order for the potential benefit of new paratuberculosis vaccine candidates to be assessed, experimental infection should lead to measurable shedding of the bacterium in the feces, as it is the only antemortem quantitative assessment of infection status.

Various animal models for paratuberculosis have been developed and studied in a wide range of ruminant species, as well as rodents (Hines et al., 2007; Begg and Whittington, 2008). The majority of these studies have utilized oral inoculation to simulate uptake of the bacteria in the field via fecal–oral transmission. Oral inoculation of calves has successfully resulted in a subclinical infection model but has rarely resulted in clinical disease. Attempts to develop a calf model of infection that will transition from subclinical to clinical infection in a suitable period of time (12 months or less) have failed (Stabel et al., 2003; Uzonna et al., 2003; Waters et al., 2003). Further, it was

* Corresponding author. USDA-ARS, National Animal Disease Center, 2300 Dayton Rd., Ames, IA 50010, United States. Tel.: +1 515 663 7304; fax: +1 515-663-7458.
*E-mail address:* judy.stabel@ars.usda.gov (J.R. Stabel).

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recently demonstrated that experimental infection studies of longer duration (35–54 months) also failed to result in clinical disease (Stewart et al., 2007). In the present study, oral infection of neonatal calves with *Mycobacterium avium* subsp. *paratuberculosis* was compared with alternate approaches such as intraperitoneal inoculation and dexamethasone treatment of calves prior to oral infection that might serve to shorten the asymptomatic period and expedite the appearance of clinical signs.

## 2. Materials and methods

### 2.1. Animals

Neonatal Holstein dairy calves were obtained from herds in Minnesota at 1–2 days of age. The farms were status level 4 herds enrolled in the Voluntary Bovine Johne's Control Program, with no reportable incidence of Johne's disease in the last 4–5 years and a 99% probability that they were free of paratuberculosis. Calves were housed in Biosafety Level-2 containment barns for the duration of the study with control calves housed in a separate barn. Standard commercial milk replacer (Land O Lakes, Shoreview, MN) was fed 2× per day at 10 h intervals during the acclimation and experimental infection periods. At 6 weeks of age, calves were weaned onto calf starter (Kent Feeds, Muscatine, IA) and then gradually switched over to a mixed pelleted ration of corn, wheat mids, and soybean meal (Mid-State Milling, State Center, IA) and hay cubes for the remainder of the study. Fecal samples were collected on 2 consecutive days prior to initiation of the study, followed by sampling on days 7, 14, 28, and monthly thereafter for 12 months.

### 2.2. Experimental infection of calves

Treatment groups consisted of: (1) Control noninfected (Control), $n = 4$; (2) Oral, $n = 4$; (3) Oral/dexamethazone (Oral/DXM), $n = 4$; (4) Intraperitoneal (IP), $n = 4$; and (5) Oral/mucosal (Oral/M), $n = 3$. The oral group was fed milk replacer (2 l) containing on average $1 \times 10^{11}$ live *M. avium* subsp. *paratuberculosis*, low-passage strain K-10, 2× per day for 14 consecutive days. The Oral/DXM group were treated exactly the same as the oral group except the calves were administered 0.25 mg/kg BW dexamethasone (Azium, Schering Corp., Kenilworth, NJ) IV for 3 days prior to bacterial challenge and on days 28 and 56 post-challenge. Intraperitoneal inoculation of calves was performed on days 0, 7, 14, and 21 of the study. A 3 mm incision was made in the skin and the fascia covering the oblique abdominal muscle. A teat cannula was used to penetrate the final layers of muscle and peritoneum and deposit the bacteria directly into the peritoneal cavity in a 1 ml volume ($1 \times 10^{11}$ cfu) with a syringe. Calves in the IP group were fed milk replacer (2 l) 2× per day without bacteria and weaned onto solid diets at the same time points as calves in other treatment groups. The Oral/M calves were inoculated by feeding milk replacer containing $2.6 \times 10^{12}$ live *M. avium* subsp. *paratuberculosis* obtained by scraping the ileal mucosa from a clinically infected cow. Calves were dosed on days 0, 7, and 14 of the study. All procedures performed on the animals were approved by the Institutional Animal Care and Use Committee (National Animal Disease Center (NADC), Ames, Iowa).

### 2.3. Bacteria

*M. avium* subsp. *paratuberculosis*, strain K-10, was obtained as a gift from Dr. Vivek Kapur, University of Minnesota, as a frozen stock. It was characterized as "low-passage" by nature of its historical background across laboratories. The bacteria were passaged a total of 4 times by expansion in Middlebrook 7H9 medium supplemented with OADC (BD, Franklin Lakes, NJ) and 2 mg/ml mycobactin J (Allied Monitor, Fayetteville, MO) and harvested in the log phase of growth (0.2–0.4 $Abs_{540nm}$). The bacteria were pelleted by centrifugation at 7500 rpm, washed with PBS 2×, and then resuspended in PBS to an approximate concentration of $1 \times 10^{10}$–$10^{11}$ cfu/ml. Bacteria were briefly sonicated (Tekmar, Cincinnati, OH) to reduce clumping prior to experimental inoculation. The bacteria were added to the milk replacer fed to calves assigned to the Oral and Oral/DXM treatment groups at each morning and evening feeding for 14 consecutive days. The K-10 strain stock was also used for the intraperitoneal inoculation of calves. The clinical isolate of *M. avium* subsp. *paratuberculosis* was obtained from the ileum of a clinical cow (cow 509) at necropsy that had shed high numbers of *M. avium* subsp. *paratuberculosis* in the feces. The clinical isolate was validated as a cattle strain by MLSSR typing. After flushing the tissue with PBS (0.15 M, pH 7.4) a mid-line incision was made to open up the tissue and the mucosa scraped using a sterile scalpel blade. Aliquots of the mucosa (20 g) were dispensed into 50 ml sterile conical tubes and either prepared immediately for use in the study or frozen at −20 °C for future use. The sample volume was brought up to 30 ml with PBS containing penicillin (200,000 IU/l) and chloramphenicol (200 mg/l) and samples were incubated for 30 min at room temperature. After incubation, samples were homogenized (Ultra-Turrax T25, IKA, Wilmington, NC), brought up to a final 40 ml volume and added to the milk replacer at the morning feeding.

The number of viable *M. avium* subsp. *paratuberculosis* in the inocula preparations was determined by performing serial 10-fold dilutions of the stocks in PBS, followed by plating onto BBL™ Herrold's Egg Yolk Agar Slants (HEYM) with mycobactin J, amphotericin, nalidixic acid, and vancomycin (Becton Dickinson and Co., Sparks, MD) in duplicate, and culturing for 12 weeks at 39 °C. Total colony counts were performed for each dilution and the average count obtained for the most limiting dilution was used to calculate the final concentration of the stock bacteria. Viable bacteria recovered on HEYM averaged between $1 \times 10^{10}$ and $1 \times 10^{11}$/ml for both the K-10 strain and the clinical isolate. Approximate dosages of viable *M. avium* subsp. *paratuberculosis* based upon these figures were $3.6 \times 10^{12}$ for Oral and Oral/DXM calves; $4 \times 10^{11}$ for IP calves; and $8 \times 10^{12}$ for Oral/M calves during the study.

### 2.4. Culture of M. avium subsp. paratuberculosis from feces and tissues

Fecal samples (2 g) were processed by a centrifugation and double-decontamination method previously described (Stabel, 1997). Decontaminated samples (200 μl) were dispensed onto HEYM agar slants in replicates of 4 and incubated for 12 weeks at 39 °C. Culture of tissue samples was performed on HEYM utilizing 2 different methods (Stabel et al., 2003; Whittington et al., 1999). Not all tissues were available for comparison of the 2 tissue culture methods. Sections of duodenum, jejunum, ileum and their associated lymph nodes were obtained at necropsy. Sections of spiral, transverse, and descending colon as well as the spleen, ileocecal valve, hepatic and iliac lymph nodes were also taken. Portions of each tissue were weighed and homogenized in 0.75% hexadecylpyridinium chloride solution (25 volumes) by use of a stomacher for 1 min and allowed to stand overnight at room temperature to decontaminate the cultures (Stabel et al., 2003). Sediments from individual tissue homogenates (100 μl) were inoculated onto HEYM slants, 4 tubes per sample. After 12 weeks of incubation at 39 °C, viable organisms were determined by counting the number of colonies on the agar slants. The method of Whittington et al. (1999) was similar but reduced the incubation time of tissues in 0.75% HPC to 3 h, followed by centrifugation of samples at 2500 rpm for 20 min, decantation of supernatant, and resuspension of the pellets in 1 ml of antibiotic cocktail containing 100 μg/ml of vancomycin, naladixic acid, and amphotericin for 24 h at 39 °C. Homogenates were inoculated (200 μl) onto HEYM slants in duplicate, incubated at 39 °C for 12 weeks and colony counts were determined.

### 2.5. PCR analyses of M. avium subsp. paratuberculosis in feces and tissue

DNA was extracted from fecal samples using a previously described method (Stabel and Bannantine, 2005) and from selected intestinal tissues and the associated lymph nodes obtained at necropsy using a commercial kit (Qiagen, Valencia, CA). DNA was amplified by using a modified nested PCR assay for the target gene, ISMap02 (Stabel and Bannantine, 2005). The primer sequences for the outside amplification reaction were 5′-GCACGGTTTTTCGGATAACGAG-3′ (forward primer) and 5′-TCAACTGCGTCACGGTGTCCTG-3′ (reverse primer). The first round of PCR was run using a conventional thermal cycler (Peltier Thermal Cycler, MJ Research, Waltham, MA). Samples were run in triplicate in 96-well plates followed by a second amplification performed using real-time PCR (7500 Real-time PCR System, Applied Biosystems, Foster City, CA). Primers nested within the first set were 5′-GCACGGTTTTTCGGATAACGAG-3′ (forward primer) and 5′-AACCGACGCCGCCAATACG-3′ (reverse primer) for this second amplification. Negative controls consisted of reaction mixture alone (negative control), and fecal DNA from a known negative source previously identified as being negative on PCR. Positive controls consisted of genomic DNA from either strain 19698 or strain K-10 and fecal DNA from an infected cow that was confirmed by PCR.

### 2.6. Histopathologic lesions and acid-fast staining procedures

Sections of tissue were fixed by immersion in neutral-buffered 10% zinc formalin. Tissues were routinely processed, embedded in paraffin, cut at 4–6 μm, and stained with hematoxylin and eosin (HE). Adjacent sections were cut from blocks containing tissues with lesions suggestive of paratuberculosis and stained by the Ziehl-Neelsen technique to visualize acid-fast bacteria. Microscopic lesions were staged (I–IV) according to modified criteria from Palmer et al. (2007).

### 2.7. Genotyping analyses

Four short-sequence repeat (SSR) loci in the *M. avium* subsp. *paratuberculosis* genome (Locus 1, Locus 2, Locus 8, Locus 9) with the highest indices of diversity were chosen for this study (Amonsin et al., 2004; Harris et al., 2006). Individual colonies of *M. avium* subsp. *paratuberculosis* that were recovered on HEYM agar slants from the feces of experimentally infected calves in this study were picked and grown in M7H9 broth supplemented with 0.05% Tween 20, OADC, and 2 mg/ml mycobactin J. Aliquots of the original bacterial inocula (either the laboratory-adapted strain or the clinical isolate) that were used to infect the experimental calves were also plated onto HEYM and multiple colonies were selected for SSR analysis. Genomic DNA was extracted and each locus was amplified by PCR as described (Harris et al., 2006). PCR products were then purified and sequenced using standard dye terminator chemistry on an automated DNA sequencer (CEQ 8000, Beckman Coulter, Fullerton, CA). The number of tandem repeats at each locus was determined, and allele numbers assigned to reflect the number of copies represented in the SSR sequence for each locus. Multilocus SSR types (MLSSR) were then determined on the basis of the unique combination of alleles for each locus. The frequency of occurrence of MLSSR type was calculated as a percentage of the number of genotypes divided by the total number of isolates typed.

## 3. Results

Fecal culture data demonstrated that calves in the oral inoculation groups experienced shedding on days 7, 14, 21, and 28, indicative of "pass-through" shedding that is typically observed after large oral boluses of bacteria are administered (Table 1). Shedding was minimal (<5 cfu/slant) and infrequent over the remainder of the study for calves in the Oral, Oral/DXM, and IP treatment groups. However, calves in the Oral/M group shed high numbers of bacteria up to 4 months post-inoculation (1–150 cfu/slant). By 4 months post-infection, shedding was significant only in 1 of the 3 calves (79 cfu/slant), followed by sporadic shedding thereafter for all 3 Oral/M calves.

Fecal PCR results did not precisely align with fecal culture results as positive PCR signals were attained more frequently over the course of the study as compared to

**Table 1**
Culture results for fecal samples from neonatal calves experimentally infected with *M. avium* subsp. *paratuberculosis*.

| Treatment | Time after infection (days) | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 7 | 14 | 21 | 28 | 60 | 90 | 120 | 150 | 180 | 210 | 240 | 270 | 300 | 330 | 360 |
| Control | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 |
| Oral | 1/4 | 1/4 | 0/4 | 0/4 | 0/4 | 1/4 | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 |
| IP | 3/4 | 1/4 | 0/4 | 0/4 | 0/4 | 1/4 | 1/4 | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | 1/4 | 0/4 | 0/4 |
| Oral/DXM | 1/4 | 0/4 | 0/4 | 1/4 | 1/4 | 0/4 | 1/4 | 1/4 | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 |
| Oral/Mucosa | 1/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 | 0/3 | 2/3 | 1/3 | 1/3 | 1/3 | 1/3 | ND | 1/3 |

Values are expressed as the number of calves with fecal samples positive for the culture of *M. avium* subsp. *paratuberculosis* per total number of calves in the treatment group. Fecal samples were cultured on Herrold's Egg Yolk Medium for 12 weeks.
ND = Not determined.

**Table 2**
Results for detection of ISMap02 target gene by nested PCR in fecal samples from neonatal calves experimentally infected with *M. avium* subsp. *paratuberculosis*.

| Treatment | Time after infection (days) | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 7 | 14 | 21 | 28 | 60 | 90 | 120 | 150 | 180 | 210 | 240 | 270 | 300 | 330 | 360 |
| Control | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 |
| Oral | 4/4 | 4/4 | 3/4 | 2/4 | 2/4 | 4/4 | 0/4 | 1/4 | 1/4 | 1/4 | 0/4 | 3/4 | 0/4 | 2/4 | 2/4 |
| IP | 0/4 | 0/4 | 1/4 | 1/4 | 1/4 | 1/4 | 1/4 | 0/4 | 1/4 | 3/4 | 2/4 | 1/4 | 0/4 | 2/4 | 2/4 |
| Oral/DXM | 4/4 | 4/4 | 0/4 | 1/4 | 3/4 | 3/4 | 3/4 | 1/4 | 0/4 | 0/4 | 2/4 | 2/4 | 1/4 | 1/4 | 0/4 |
| Oral/Mucosa | ND | 1/3 | 3/3 | 2/3 | 1/3 | 1/3 | 2/3 | 1/3 | 1/3 | 1/3 | 0/3 | 0/3 | 0/3 | ND | 0/3 |

Values are expressed as the number of calves with fecal samples positive for the ISMap02 target gene per total number of calves in the treatment group.
ND = Not determined.

recovery of viable colonies by culture (Table 2). There was a higher degree of fecal PCR positives noted within 90 days of the study, a finding that was comparable to the fecal culture results. Interestingly, positive PCR signals were observed most frequently for Oral, Oral/DXM, and Oral/M calves in the first 120 days after challenge and to a lesser extent (1/4 calves) for the IP group. Positive results for IP calves began to appear more frequently (50–75% of calves) later in the study, around 210 days post-infection. Similarly, detection of fecal shedding of *M. avium* subsp. *paratuberculosis* by PCR was also observed in Oral and Oral/DXM calves during the latter part of the study but not in Oral/M calves.

Of the 22 tissues per calf, only 46% of tissues were positive on either culture method used. The majority of the positive tissues were identified by method 1, although colonies were small and pinpoint and difficult to quantify. Random colonies were scraped from the agar slant of positive samples and confirmed as *M. avium* subsp. *paratuberculosis* by PCR to allay doubts to the nature of these colonies. Method 2 differed substantially from method 1 as it utilized more tissue (2 g versus weighed portions of tissue averaging 500 mg), subjected the tissue homogenate to reduced time in HPC, and required overnight incubation in an antibiotic cocktail. Results demonstrated that of the 133 positive tissues, only 21% of these tissues were positive by both methods, indicating a significant impact of culture method on the recovery of viable *M. avium* subsp. *paratuberculosis* from tissues. The most frequent alignment of positive tissues occurred when tissues were cultured from a heavily infected calf in the Oral/M group. No other pattern of positive culture results was observed. The number of colonies recovered per tissue did not differ substantially between the 2 methods. Results from the two methods of tissue culture were combined due to the complementary nature of the 2 methods.

Tissue culture results showed that all calves exposed to *M. avium* subsp. *paratuberculosis* by either oral or IP methods became infected, and colonization was noted in multiple tissue sites (Table 3). Ranking of positive tissue sites demonstrated preferential colonization of the jejunum, followed by the ileum, duodenum, and spiral colon, along with the associated lymph nodes for those tissue sites. When comparing the method of *M. avium* subsp. *paratuberculosis* inoculation, the lowest number of affected

**Table 3**
Number and rank of culture positive tissue sites obtained from calves at necropsy following oral or intraperitoneal challenge with live *M. avium* subsp. *paratuberculosis*[a].

| Tissue | Treatment | | | | |
|---|---|---|---|---|---|
| | Total | Oral | IP | Oral/DXM | Oral/M |
| Rank of major positive tissues | | | | | |
| Jejenum + LN[b](Proximal, Mid-, Distal) | 41 | 13 | 6 | 10 | 12 |
| Ileum + LN[c](Proximal, Mid-, Distal) | 23 | 4 | 6 | 4 | 9 |
| Duodenum + LN[d] | 14 | 4 | 4 | 3 | 3 |
| Spiral colon + LN[d] | 13 | 5 | 2 | 3 | 3 |
| Ileocecal valve | 10 | 2 | 4 | 2 | 2 |
| Spleen | 9 | 2 | 4 | 2 | 1 |
| Iliac LN | 8 | 3 | 2 | 2 | 1 |
| Transcending colon + LN[d] | 7 | 1 | 1 | 2 | 3 |
| Ileocecal LN | 4 | 1 | 2 | 0 | 1 |
| Descending colon + LN[d] | 4 | 2 | 0 | 1 | 1 |
| Average number of positive tissue sites | | 10.3 | 8.5 | 9.5 | 15 |

[a] Total number of tissue sites sampled per calf was 22.
[b] Jejunum had 3 sections sampled (proximal, mid-, and distal) per calf plus 3 associated lymph nodes ($n = 6$).
[c] Ileum had 3 sections sampled (proximal, mid-, and distal) per calf plus one associated lymph node ($n = 4$).
[d] When depicted as tissue + LN, the associated lymph node (LN) with that tissue site was also collected ($n = 2$ tissue sites).

**Table 4**
Results of PCR on selected tissues obtained from calves at necropsy following oral or intraperitoneal challenge with live *M. avium* subsp. *paratuberculosis*[a].

| Tissue | Treatment | | | |
|---|---|---|---|---|
| | Oral | IP | Oral/DXM | Oral/M |
| Ileocecal valve | 1/4[b] | 3/4 | 2/4 | 2/3 |
| Ileocecal LN | 1/4 | 3/4 | 1/4 | 1/3 |
| Proximal ileum | 2/4 | 2/4 | 2/4 | 3/3 |
| Mid-ileum | 1/4 | 3/4 | 0/4 | 1/3 |
| Mid-ileum LN | 1/4 | 3/4 | 0/4 | 0/3 |
| Distal ileum | 0/4 | 1/4 | 1/4 | 0/3 |
| Proximal jejunum | 3/4 | 1/4 | 3/4 | 0/3 |
| Proximal jejunal LN | 1/4 | 1/4 | 0/4 | 2/3 |
| Mid-jejunum | 0/4 | 0/4 | 1/4 | 0/3 |
| Mid-jejunal LN | 0/4 | 3/4 | ND[c] | ND |
| Distal jejunum | 0/4 | 1/4 | 1/4 | 1/3 |
| Distal jejunal LN | 1/4 | 1/4 | 1/4 | 1/3 |

[a] Twelve tissue sites were processed for PCR analysis per calf.
[b] Values are expressed as the number of calves with tissue samples positive for the ISMap02 target gene per total number of calves in the treatment group.
[c] ND = Not determined.

tissues was observed in IP calves (8.5 tissue sites positive) and the highest number for calves in the Oral/M group (15 tissue sites positive). The number of viable *M. avium* subsp. *paratuberculosis* recovered by culture was low in tissues (1–5 cfu/slant) regardless of treatment group, with the exception of one calf in the Oral/M from which high numbers of *M. avium* subsp. *paratuberculosis* were recovered from the proximal jejunum, proximal jejunal lymph node, duodenum, and spiral colon (78–89 cfu/slant). The hepatic LN was culture positive for only 2 calves, 1 in the IP treatment and 1 in the Oral/M group (data not shown).

The total number of tissue sites positive by PCR per treatment group ranged between 7 and 11 for the Oral/M and IP groups, respectively (Table 4). Fewer tissues were processed for PCR than culture (12 versus 22) due to cost factors, so efforts were focused on sections of the small intestine as the primary target tissue. Of the 12 sites sampled, the IP calves had the greatest number of positive sites (11) as compared to 7–8 positive sites for the orally inoculated calves. The mid-ileum and the ileocecal valve, and their associated lymph nodes were recorded most frequently as positive by PCR for the IP calves. In contrast, the proximal jejunum and proximal ileum were the two tissues most frequently positive for calves receiving *M. avium* subsp. *paratuberculosis* by the oral route.

Lesions were most pronounced within tissues from Oral/DXM calves and secondarily for the Oral group. Three of the 4 calves in the Oral/DXM group had microscopic lesions commonly associated with paratuberculosis, consisting of multifocal infiltrates of low to moderate numbers of epithelioid macrophages and multinucleated giant cells expanding the lamina propria and extending through the submucosa into the submucosal lymphoid follicles. Such lesions were most common in the ileum and ileocecal valve, although similar lesions were seen less commonly in the proximal, mid-, and distal jejunum, cecum, ascending and descending colon. Similar infiltrates were also seen in sections of lymph nodes associated with the ileum, ileocecal valve, colon and jejunum. In 1 of the 4 Oral/DXM calves lesions were limited to a focal granuloma in the colic lymph node. Lesions consistent with paratuberculosis were seen in only 1 of 4 Oral calves (Fig. 1). These lesions were most common in the ileum and ileocecal valve, although similar lesions were seen less commonly in the proximal, mid-, and distal jejunum, cecum, and no lesions were found in the large intestine. Infiltrates of epithelioid macrophages and giant cells were also seen in sections of lymph nodes associated with the ileum, ileocecal valve, and jejunum. Similarly, these lesions were found in 1 of 3 calves in the Oral/M group, with lesions limited to sections of the ileum and ileocecal valve. Small, focal to multifocal aggregates of epithelioid macrophages with rare multinucleated giant cells were seen in lymph nodes of the jejunum, ileum, ileocecal valve, and colon. Lesions associated with paratuberculosis were observed in only 2 of 4 IP calves. In one calf, multifocal granulomas composed of epithelioid macrophages and multinucleated giant cells were limited to the lamina propria and submucosa of the ileocecal valve (Fig. 2). Lesions in the

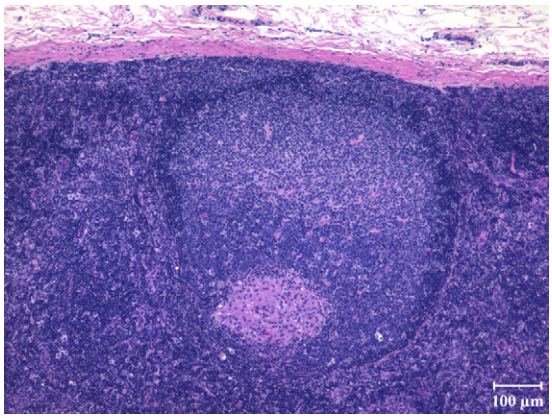


**Fig. 1.** Representative section of the ileocecal lymph node obtained from a calf experimentally infected via oral inoculation with a clinical isolate of *M. avium* subsp. *paratuberculosis*. Sections were stained with hematoxylin and eosin. Note small, focal aggregate of epithelioid macrophages within lymph node follicle. HE, Bar = 100 µm.

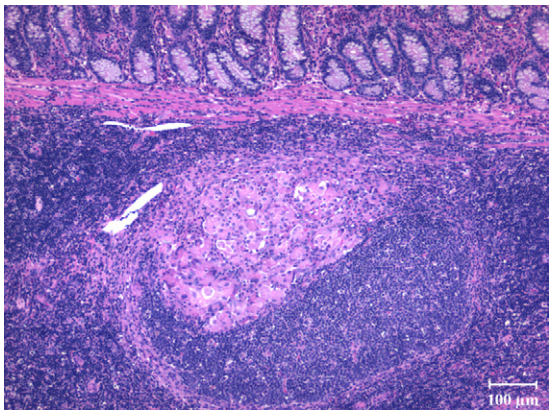


**Fig. 2.** Representative section of the ileocecal valve obtained from a calf experimentally infected via intraperitoneal inoculation with *M. avium* subsp. *paratuberculosis*. Note small focal granuloma in the submucosal lymphoid tissue. HE, Bar = 100 µm.

**Table 5**
Analyses of single short repeat loci for two isolates of *M. avium* subsp. *paratuberculosis* in the original inocula and after recovery from fecal samples of experimentally infected calves.

| Isolate | Source | SSR Loci | | | |
|---|---|---|---|---|---|
| | | Locus 1 | Locus 2 | Locus 8 | Locus 9 |
| Strain K-10 | Inoculum | >14 | 10 | 5 | 5 |
| | Output | >14 | 10 | 5 | 5 |
| Clinical isolate | Inoculum | 7 | 9 | 4 | 3 |
| | | 7 | 10 | 4 | 3 |
| | Output | 7 | 9 | 4 | 3 |
| | | 7 | 10 | 4 | 3 |

second calf were limited to the proximal jejunum and ileocecal valve and characterized by multifocal aggregates of low to moderate numbers of epithelioid macrophages and multinucleated giant cells within the lamina propria and submucosa. Small, focal to multifocal aggregates of epithelioid macrophages with rare multinucleated giant cells were seen in the lymph nodes of the ileum and ileocecal valve. Acid-fast organisms were not detected within the tissues of calves, regardless of bacterial strain used for inoculation or the route of inoculation.

Analysis of the *M. avium* subsp. *paratuberculosis* isolates recovered from the infected calves indicated that there was no variation in the MLSSR types as compared to the original inoculum. However, there were distinct differences in genotype between the low-passage strain K-10 and the clinical isolate of *M. avium* subsp. *paratuberculosis* used to inoculate the calves in the study. Strain K-10 demonstrated the genotype of >14-10-5-5. Infection route (oral or IP) did not influence the genotype of *M. avium* subsp. *paratuberculosis* shed in the feces of these calves. The *M. avium* subsp. *paratuberculosis* isolate obtained from the mucosal scrapings of a clinical cow yielded two similar MLSSR types; 7-9-4-3 and 7-10-4-3, suggesting that 2 strains of *M. avium* subsp. *paratuberculosis* were present in the ileum of the infected cow. Although the number of samples available for analysis was limited due to infrequent fecal shedding by calves in the study, it was noted that MLSSR 7-10-4-3 increased in frequency in one animal in the Oral/M from 7.7% at 2 months of infection to 39% at 4 months of infection (Table 5).

## 4. Discussion

Using an experimental infection model provides a platform for conducting controlled experiments with the exact knowledge of the level of exposure on an individual animal basis. This allows for more comprehensive studies to evaluate vaccines, therapeutics, or antimicrobials. A calf experimental infection model for *M. avium* subsp. *paratuberculosis* is particularly important because of the high prevalence demonstrated in the dairy cattle population in the US (Ott et al., 1999). A number of studies have reported successful experimental infection of neonatal calves, noting some degree of tissue colonization or microscopic lesions as indicators of infection, however, the success of the model is influenced by route, dose, and source of the inoculum.

The majority of studies in cattle have effectively utilized an oral route of infection, either suckling or gastric gavage, in an attempt to simulate the natural exposure of the neonate to *M. avium* subsp. *paratuberculosis* through ingestion of contaminated feces or milk from infected dams (Gilmour et al., 1965; Larsen et al., 1973; Uzonna et al., 2003; Stabel et al., 2003; Sweeney et al., 2006), although intratonsilar inoculation was found to be effective in establishing subclinical infection as well (Waters et al., 2003). Some early studies reported an endpoint of clinical disease (Rankin, 1961; Larsen et al., 1973), utilizing an oral route of infection, however, calves were maintained for periods ranging from 4 to 6 years.

Due to an inability to quantify the number of viable bacteria in the mucosal scrapings prior to inoculation there was some disparity in the dosage of calves between treatment groups, yielding up to 20-fold differences in the total dose of *M. avium* subsp. *paratuberculosis* received. This may explain some of the differences noted between the treatment groups in the present study. Feeding the bacteria in the milk in repeated doses over a 14-day period to calves in the oral groups did not seem to increase infectivity compared to previous studies using a more restricted dosing schedule (Stabel et al., 2003; Uzonna et al., 2003; Waters et al., 2003). Similarly, clinical signs were not demonstrated in other studies utilizing a bacterial dosing schedule over periods ranging from 10 days to 10 weeks (Gilmour et al., 1965; Saxegaard, 1990; Koo et al., 2004).

Administration of dexamethasone to calves prior to oral inoculation with *M. avium* subsp. *paratuberculosis*, strain K-10, did not result in substantial differences in fecal shedding or tissue colonization compared to oral inoculation alone. However, the greater degree of lesions observed in tissues from the Oral/DXM calves suggests that pharmacologic modulation prior to bacterial challenge altered the host's ability to contain the inflammatory responses invoked by the pathogen. A prior report in golden hamsters demonstrated increased colonization of tissues with *M. avium* subsp. *paratuberculosis* in animals dosed with dexamethasone prior to infection, however, pathologic assessment of lesions in tissues was not performed (Larsen and Miller, 1978). In the present study, Oral/DXM calves had reduced γδ T cells in the peripheral blood compared to calves in other treatments (data not shown). Dexamethasone treatment is hypothesized to result in a redistribution of γδ T cells from the peripheral blood to epithelial tissues in cattle (Burton and Kehrli, 1996), where they maintain a role in inflammatory responses (Kaufman, 1996). The predominance of lesions within the tissues of Oral/DXM calves suggests that experimental immunosuppression prior to infection may result in the inability of the host to retard localized damage due to colonization.

Oral inoculation of calves with the clinical cow isolate of *M. avium* subsp. *paratuberculosis* resulted in a greater number of tissue sites that were positive, as well an increase in level of infectivity within the tissues for at least 1 of the 3 calves. Interestingly, this increase in tissue colonization did not correlate with microscopic lesions. The appearance of lesions in the intestinal tissues in cows

with paratuberculosis appears to be highly correlated with clinical disease, however, tuberculoid lesions have been reported in asymptomatic cattle as well (Brady et al., 2008). Lesions associated with lymph nodes have been found more frequently in experimentally infected cattle and cattle in the early stages of natural infection (Sigurethardóttir et al., 2004; González et al., 2005). In the present study, multifocal granulomas were observed in the lymph nodes of the jejunum, ileum, ileocecal valve, and colon, of calves in each treatment group, regardless of mode of infection, indicating that this is a hallmark characteristic of subclinical infection.

The greater number of positive tissue sites and the more protracted sequence of fecal shedding noted for Oral/M calves would indicate that inoculation with a clinical isolate of *M. avium* subsp. *paratuberculosis* is more efficacious than a laboratory-adapted strain in establishing infection. No clinical signs of disease were observed in the present study, however, use of the mucosal scrapings from a clinically infected animal has resulted in presentation of clinical signs in sheep and goats (Gwozdz et al., 2000; Stewart et al., 2006). Although clinical disease has been reported in some studies in which an isolate of *M. avium* subsp. *paratuberculosis* has been subjected to limited passages (<3) in vitro, it is not known to what degree bacterial virulence is affected by multiple passages (Gilmour et al., 1978). A recent study comparing a laboratory-adapted strain and a clinical isolate of *M. avium* subsp. *paratuberculosis* demonstrated a significant difference in the pattern of protein expression by the 2 strains, suggesting that in vitro propagation of the bacterium prior to use in an experimental infection may affect its degree of infectivity (Radosevich et al., 2007).

The intraperitoneal route of infection has rarely been used for calves and those few cases have been infection models for parasites, not for bacterial pathogens (Gallie and Sewell, 1981; Lindsay et al., 1990). There is no record in the published literature of intraperitoneal inoculation of calves with *M. avium* subsp. *paratuberculosis* as an experimental model. However, a number of studies have successfully utilized the IP method of inoculation in the mouse model to achieve infection with *M. avium* subsp. *paratuberculosis* (Chiodini and Buergelt, 1993; Veazey et al., 1996). In the present study, the IP calves had similar numbers of culture positive tissue sites to Oral and Oral/DXM calves, differing significantly only from the Oral/M group. Interestingly, 2 of the 4 IP calves demonstrated a positive antibody titer on a commercial ELISA (data not shown), indicative of a switch toward Th2-mediated immunity associated with the later stages of infection. This was the only treatment group with positive antibody responses during the study, suggesting that further investigation into this type of model is warranted.

Analysis of the *M. avium* subsp. *paratuberculosis* isolates recovered from the infected calves indicated that there was no variation in the MLSSR types as compared to the inoculum. *M. avium* subsp. *paratuberculosis* isolates from calves infected with strain K-10 had identical MLSSR types to the original K-10 inoculum, genotype >14-10-5-5 as previously reported (Harris et al., 2006). Similarly, *M. avium* subsp. *paratuberculosis* recovered from calves receiving the clinical isolate exhibited the same two genotypes as the original isolates. Therefore, these data support the hypothesis that *M. avium* subsp. *paratuberculosis* SSR loci remain stable during the initial stages of disease, and thus are appropriate for use in investigating methods of transmission between animals. Interestingly, the recovery of 2 *M. avium* subsp. *paratuberculosis* genotypes from the mucosa of the clinical cow suggests that a mixed infection had occurred in the field. This is consistent with an earlier survey of US dairy cattle in which several SSR genotypes were identified both within herds and within individual animals in a herd (Harris et al., 2006).

In summary, the experimental methods of infection did not result in clinical signs of disease within the 12-month period of the study, yet there were a significant number of affected tissues, either by culture, PCR, or microscopic analyses. Although the IP method did result in infection, the oral method was the most consistent and, perhaps, natural approach of experimental exposure to *M. avium* subsp. *paratuberculosis*. These data suggest that oral inoculation remains the most effective method of experimental infection for *M. avium* subsp. *paratuberculosis* in calves, and that inoculation with a clinical isolate may induce greater infectivity.

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